Supporting Information for:

High Performance Near-Infrared Fluorescent Secondary Antibodies for Immunofluorescence

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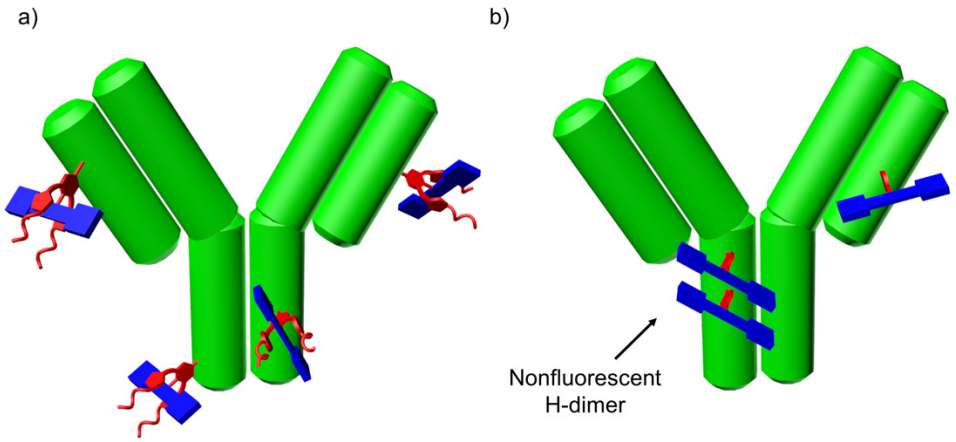
1. General Concept Underlying Difference in Antibody Labeling



2'Ab-s775z 2'Ab-CW800 or 2'Ab-DyLight800

Scheme S1. Conceptual picture of goat anti-rabbit IgG antibody labeled with: a) four copies of s775z to give 2'Ab-s775z, the two red shielding arms on each blue fluorochrome ensures that the s775z NHS ester molecules react with separated lysine residues on the antibody surface. b) three copies of CW800 or DyLight800 to give 2'Ab-CW800 or 2'Ab-DyLight800, the propensity of the reactive NHS esters to self-aggregate favors dye attachment at proximal lysine positions on the IgG surface leading to a highly quenched (non-fluorescent) antibody-dye conjugate that exhibits a strong "H-dimer" peak at 710 nm in the absorption spectrum.

2. Photophysical Properties

Table S1. Spectral Properties of Free Dyes in PBS (pH 7.4).

	s775z	**IRDye CW800®**	**DyLight800**
λ^{abs}_{max} (nm)	775	775	771
λ^{em}_{max} (nm)	794	796	798
ε ($M^{-1}cm^{-1}$)	201,000	242,000	270,000
QY	0.090	0.090	0.034

Absorbance Profiles. Absorbance spectra of dye-labeled goat anti-rabbit IgG antibody conjugates were collected in Phosphate Buffered Saline using Implen NanoPhotometer NP80 and normalized to the absorbance at 280 nm.

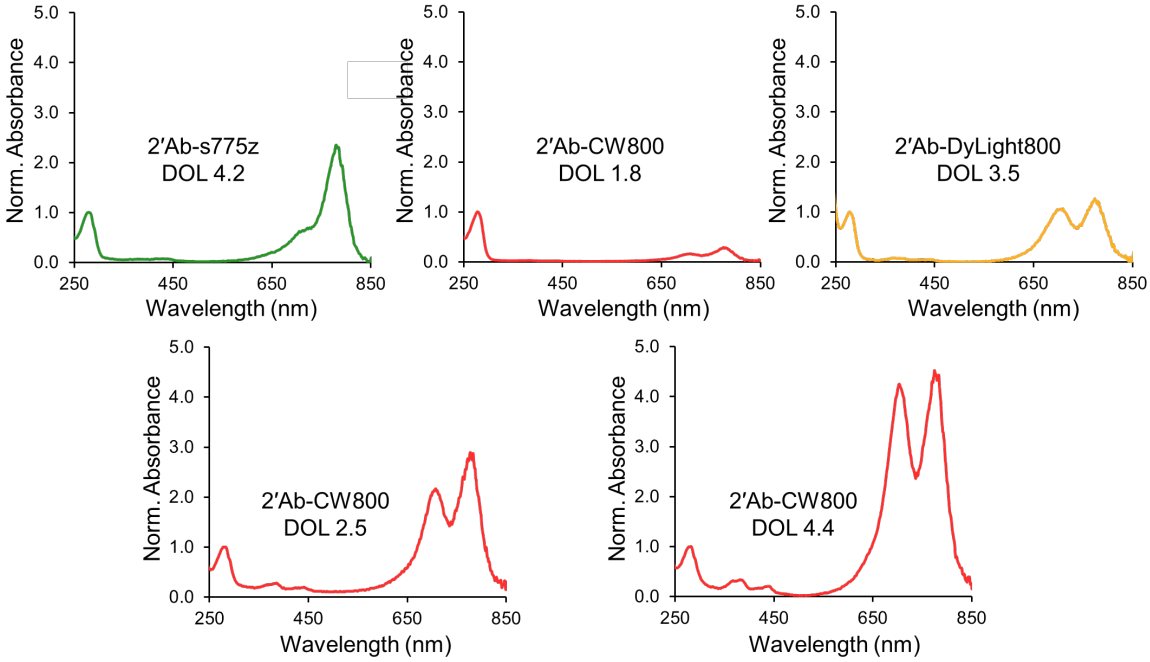


Figure S1. Absorbance spectra of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes. Antibodies labeled with CW800 (DOL 1.8) and antibodies labeled with DyLight800 (DOL 3.5) were purchased and shipped in BSA which contributes to the absorbance peak at 280 nm. DOL is degree of labeling. The top row corresponds to the three antibody conjugates that were compared in manuscript Figures 1 - 5.

Storage Stability. Absorbance spectra of 2'Ab-s775z (DOL 4.2) and 2'Ab-CW800 (DOL 4.4) were collected in Phosphate Buffered Saline using Implen NanoPhotometer NP80. Spectra in Figure S2 were acquired at 0 and 10 days after antibody conjugation and were normalized to the absorbance at 280 nm. The antibody conjugates were stored at 4 °C between data collection.

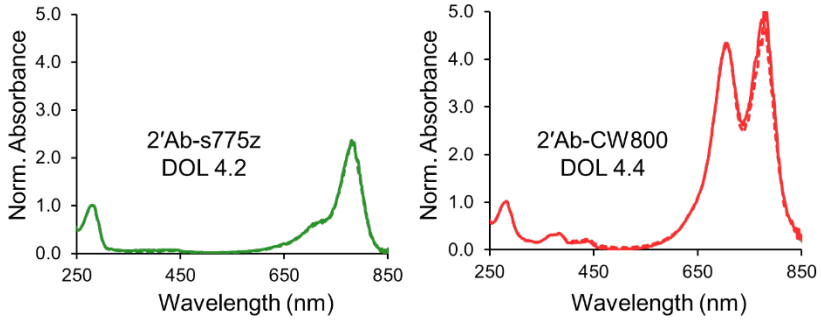


Figure S2. Absorbance spectra of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes upon storage in Phosphate Buffered Saline at 4 °C over 10 days. The solid line represents day 0, and the dashed line represents day 10. DOL is degree of labeling.

Conjugate Purity. Gel electrophoresis was used to verify that each sample of 2'Ab-s775z and 2'Ab-CW800 was a covalently bonded conjugate. In each case, a single NIR fluorescent band was observed at the expected IgG molecular weight of ~150 kDa with no measurable amount of unconjugated dye (Figure S4A).

3. Degree of Labeling (DOL)

The degree of labeling (DOL) was calculated using the maximum absorbance method with the following equation:

$$DOL = \frac{A_{max}\epsilon_{antibody}}{(A_{280}-A_{max}C_{280})\epsilon_{dye}}$$

A_{max} is the maximum absorbance due to monomer dye, A_{280} is the absorbance at 280 nm, and $\epsilon_{antibody}$ is 203,000 $M^{-1}cm^{-1}$. For 2'Ab-CW800, C_{280} is 0.03 and ϵ_{dye} is 240,000 $M^{-1}cm^{-1}$ based on manufacturer specifications. For 2'Ab-s775z, C_{280} is 0.22 based on the ratio A_{max}/A_{280} of unconjugated s775z dye and ϵ_{dye} is 201,000 $M^{-1}cm^{-1}$. **Note that C_{280} for 2'Ab-s775z is seven times higher than 2'Ab-CW800 due to additional absorption at 280 nm by the two triazole groups within the structure of the s775z dye in 2'Ab-s775z.**

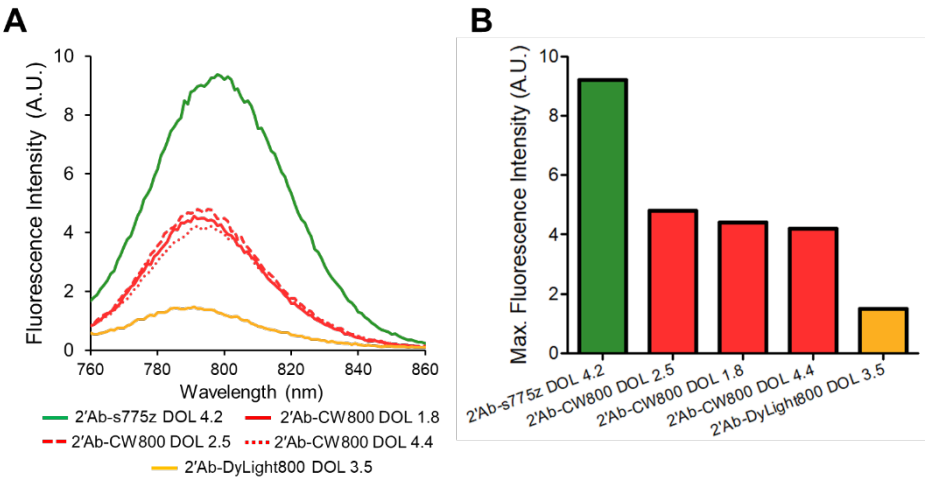


Figure S3. Fluorescence spectra for separate solutions containing equal amounts of secondary goat anti-rabbit IgG antibodies (0.035 mg/mL) labeled with different NIR dyes. (A) Fluorescence emission spectra of the separate antibody conjugates collected at excitation wavelength 745 nm. Slit width 4 nm. (B) Bar graph of the maximum fluorescence intensity value from fluorescence emission spectra. DOL is degree of labeling.

Polyacrylamide Gel Electrophoresis. Aqueous samples of purified antibodies labeled with s775z (2'Ab-s775z) or CW800 (2'Ab-CW800) were loaded into a 4-15% Mini-PROTEAN TGX precast protein gel (Bio-Rad). The gel was run at 125 V for 45 min and was then imaged with an in vivo imaging station (Ami HT Spectral Instruments Imaging) (ex: 745 nm, em: 850 nm, exposure: 5 sec, percent power: 100%, F-stop: 2, binning: small).

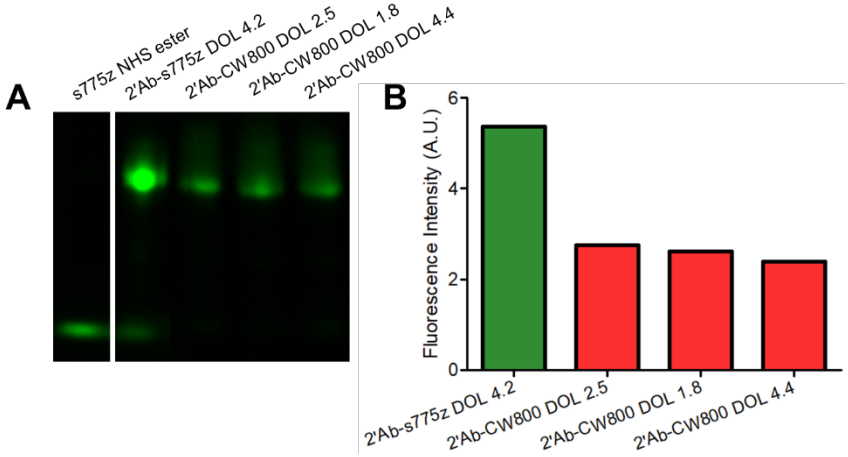
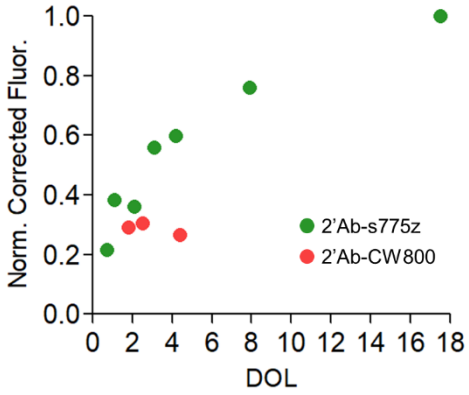


Figure S4. Polyacrylamide gel electrophoresis of separate samples of secondary goat anti-rabbit IgG antibodies labeled with different NIR dyes. (A) Fluorescent gel image of separate antibody conjugates (each 1 mg/mL) collected at excitation wavelength 745 nm and emission wavelength 850 nm. (B) Bar graph of fluorescence intensity of each fluorescent band on gel. DOL is degree of labeling. The antibody conjugates in this figure are the same as Figure S3.



Figures S5. Plot of DOL for 2'Ab-s775z and 2'Ab-CW800 versus fluorescence intensity (corrected for protein concentration and normalized relative to 2'Ab-s775z with DOL=17.5) for different bands of pure 2'Ab-s775z and 2'Ab-CW800 on a polyacrylamide gel.

4. Immunocytochemistry

4a. Single-Color Imaging of Cellular Tubulin Using 2'Ab-CW800 with Varying DOL

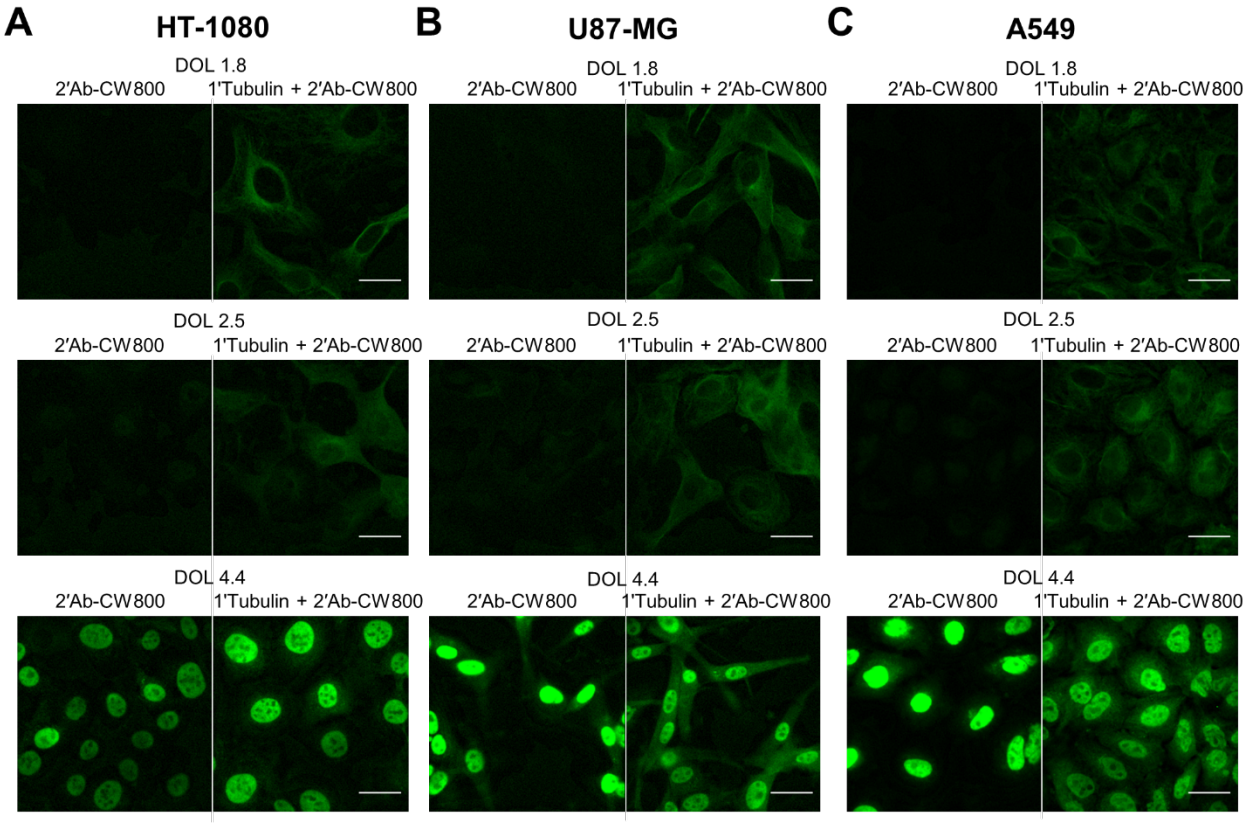


Figure S6. Comparison of secondary goat anti-rabbit IgG antibodies labeled with CW800 (2'Ab-CW800) at varying DOL for targeting to primary anti-Tubulin in fixed cells. HT-1080, U87-MG, and A549 cells were fixed, incubated with anti-Tubulin overnight at 4 °C, and incubated the following day with 2'Ab-CW800 for 2 hr at room temperature. Representative epifluorescence cell micrographs depict (A) HT-1080, (B) U87-MG, and (C) A549 cells stained with 2'Ab-CW800 without (left) or with primary anti-Tubulin (right). Scale bar = 30 μm.

4b. Single-Color Imaging of Cellular HSP72

HSP72 is a member of the 70-kDa heat shock protein family (HSP70) that is strongly expressed in the cytosol and on surface of cancer cells. HT-1080 fibrosarcoma cells are known to express high levels of HSP72 and were utilized for this imaging study. HT-1080 cells were grown in an 8-well chambered coverglass, fixed with 4% paraformaldehyde, incubated with blocking buffer, and incubated overnight at 4 °C with primary rabbit anti-HSP70/72 (1:100, Enzo Life Sciences, ADI-SPA-810). The following day, each well was incubated for 2 hr at room temperature with a different fluorescent secondary antibody, 2'Ab-s775z or 2'Ab-CW800. NIR fluorescence micrographs were acquired, and the cells stained with 2'Ab-s775z displayed 3 times higher fluorescence intensity compared to cells stained with 2'Ab-CW800 (Figure S7).

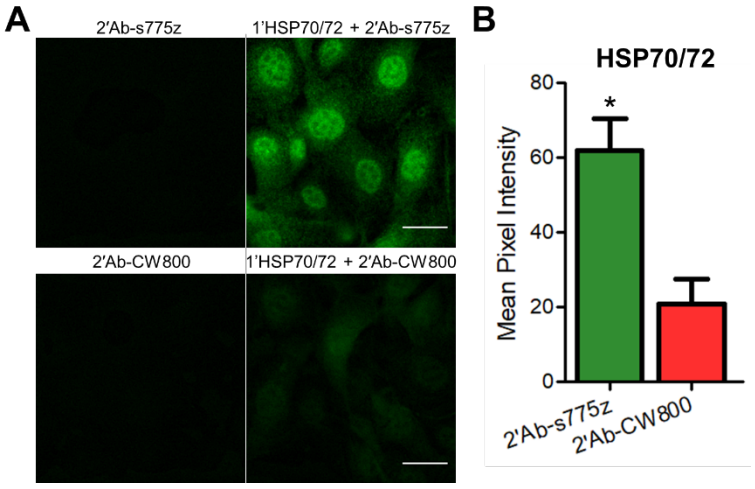


Figure S7. Comparison of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes for targeting to primary anti-HSP70/72 in fixed cells. HT-1080 cells were fixed, incubated with anti-HSP70/72 overnight at 4 °C, and incubated the following day with a fluorescent secondary antibody (2'Ab-s775z, 2'Ab-CW800) for 2 hr at room temperature. (A) Representative epifluorescence cell micrographs depict fluorescent secondary antibody without (left) or with primary anti-HSP70/72 (right). (B) The mean pixel intensity of cell micrographs where cells were incubated with primary anti-Tubulin and a fluorescent secondary antibody. Scale bar = 30 μm. p-value < 0.05 (*).

The photostability data in Figure S11B-C shows that continuous microscope irradiation of cells stained with primary anti-HSP70/72 and 2'Ab-s775z photobleached 5 times more slowly than cells stained with primary anti-HSP70/72 and 2'Ab-CW800 (Table S2). The same difference in photostability was obtained when the antibody stained cells were exposed to sunlight for 16 hr (Figure S11A).

4c. Control Immunocytochemistry Experiments

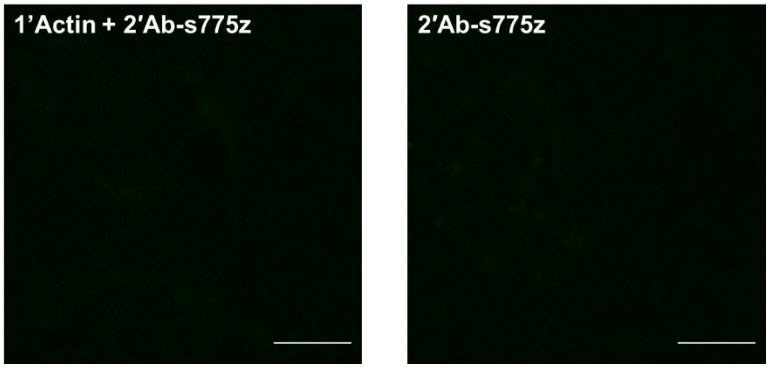


Figure S8a. Control experiments for multiplex imaging of HT-1080 cells. (left) The mismatched pair of primary mouse anti-Actin and secondary anti-rabbit (2'Ab-s775z) produced minimal cell fluorescence, confirming no cross-reactivity. (right) Cell treatment with 2'Ab-s775z alone produced minimal cell fluorescence.

Interestingly, when HT-1080 cells were incubated with secondary anti-mouse 2'Ab-FITC (alone or in the presence of primary rabbit anti-Tubulin), the resulting fluorescence micrographs indicated 2'Ab-FITC accumulation within the nucleus (Figure S8b). This non-specific localization of 2'Ab-FITC in the nucleus is very similar to the off-target behavior of 2'Ab-CW800 (DOL 4.4) in Figure S6 and likewise is attributed to the electrostatic attraction of the anionic dye-labelled conjugate to cationic histones in the nucleus. The non-specific affinity of anti-mouse 2'Ab-FITC for the nucleus is apparently weak because it is not seen when the cells are pre-incubated with primary mouse anti-Actin antibody as shown in manuscript Figure 3.

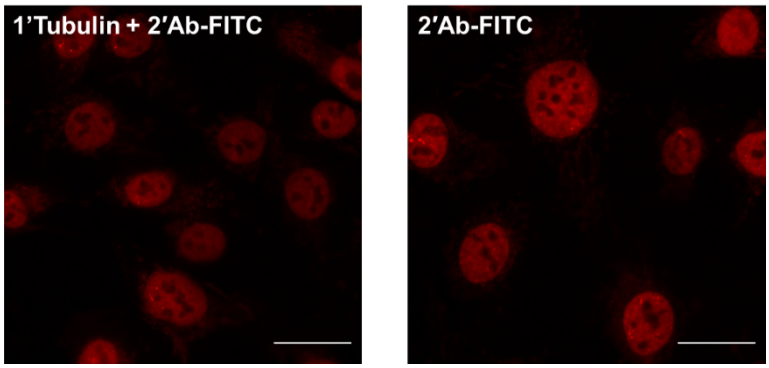


Figure S8b. Control experiments for imaging of HT-1080 cells using commercial secondary anti-mouse 2'Ab-FITC. (left) Cells incubated with 2'Ab-FITC and primary rabbit anti-Tubulin. (right) Cells incubated with 2'Ab-FITC alone. In both cases, the 2'Ab-FITC accumulated within the cell nucleus. This data is discussed above.

5. Photostability

Figure S9 shows that cells stained with 2'Ab-s775z exhibit equal or higher brightness after 15 min of continuous irradiation, than cells that were stained using 2'Ab-CW800 or 2'Ab-DyLight800 and kept in the dark (i.e., no microscope NIR irradiation).

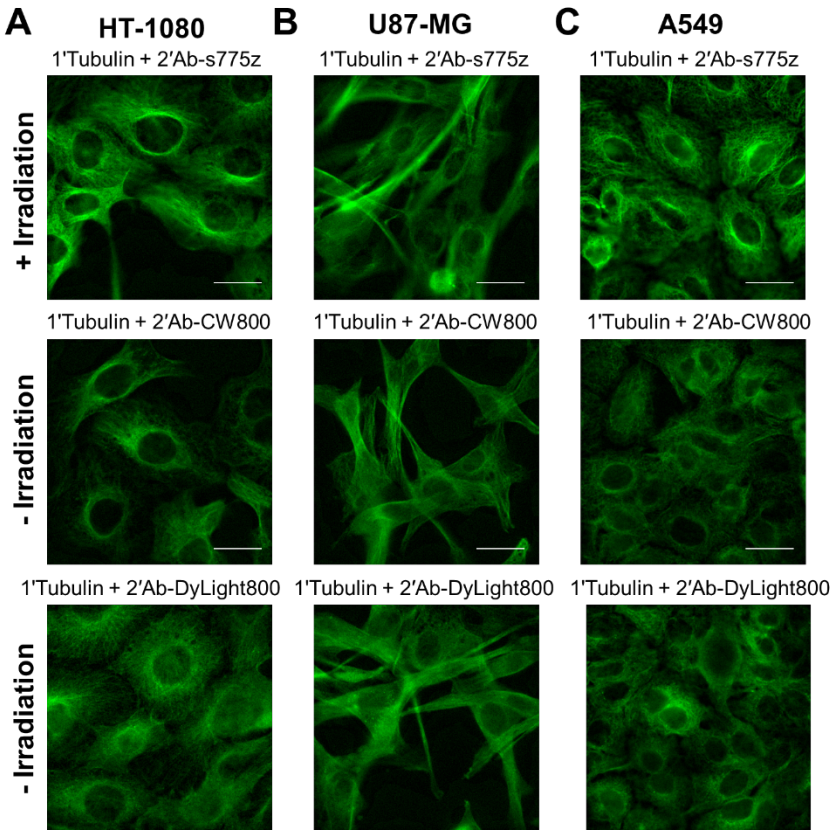


Figure S9. Photostability comparison of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes. HT-1080, U87-MG, and A549 cells were fixed, incubated with anti-Tubulin overnight at 4 °C, and incubated the following day with fluorescent secondary antibody (2'Ab-s775z, 2'Ab-CW800, 2'Ab-DyLight800) for 2 hr at room temperature. Cells incubated with 2'Ab-s775z were then continuously irradiated for 15 min with a microscope equipped with an ICG filter (ex: 769/41, em: 832/37). Representative epifluorescence cell micrographs of (A) HT-1080, (B) U87-MG, and (C) A549 cells incubated with 2'Ab-s775z and exposed to light irradiation (top row) or incubated with secondary antibodies 2'Ab-CW800 (middle row) or 2'Ab-DyLight800 (bottom row) with no irradiation. Scale bar = 30 μm.

Sunlight Irradiation Experiments. HT-1080, A549, and U87-MG cells incubated with primary anti-Tubulin and a fluorescent secondary antibody, were imaged on a Zeiss Axiovert 100 TV epifluorescence microscope equipped with an ICG filter (ex: 769/41, em: 832/37). The cells were then exposed to sunlight for 16 hr and were imaged again. Image processing for each micrograph was then conducted using ImageJ2 software with a 200-pixel rolling ball radius background subtraction. Procedure was repeated with HT-1080 cells incubated with primary anti-HSP70/72 and a fluorescent secondary antibody. The results in Figure S10 shows that cells stained with 2'Ab-s775z exhibit equal or higher brightness after 16 hr of continuous sunlight, than cells that were stained using 2'Ab-CW800 or 2'Ab- DyLight800 and kept in the dark (i.e., no sunlight).

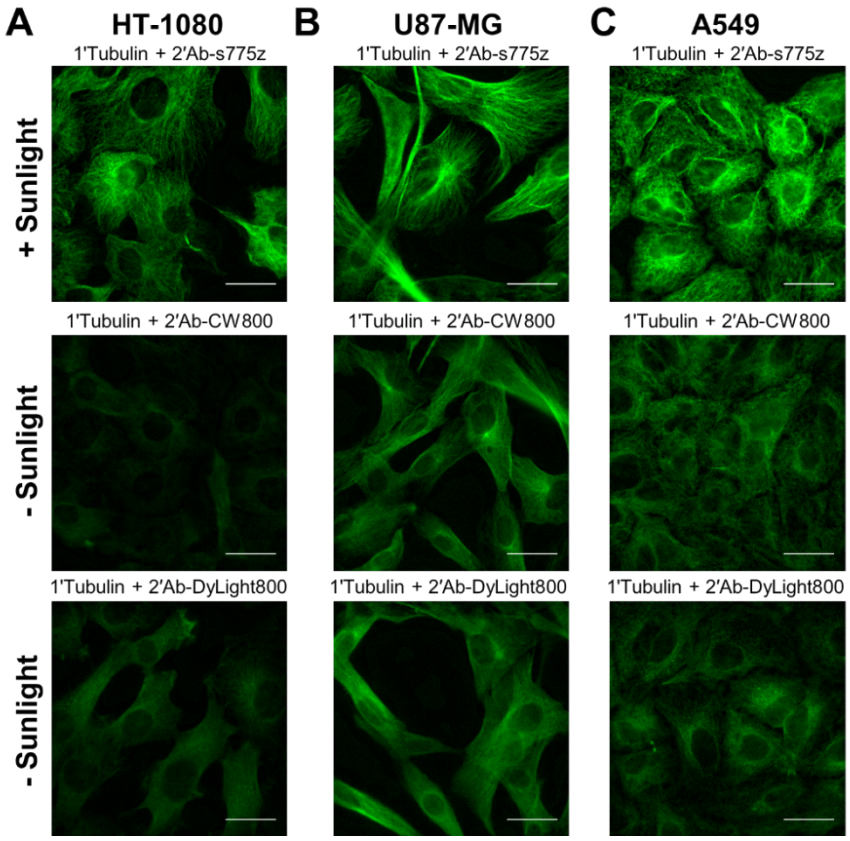


Figure S10. Photostability comparison of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes for targeting to primary anti-Tubulin in fixed cells. HT-1080, U87-MG, and A549 cells were fixed, incubated with anti-Tubulin overnight at 4 °C, and incubated the following day with fluorescent secondary antibody (2'Ab-s775z, 2'Ab-CW800, 2'Ab-DyLight800) for 2 hr at room temperature. Representative epifluorescence cell micrographs of (A) HT-1080, (B) U87-MG, and (C) A549 cells incubated with 2'Ab-s775z (top row) and exposed to sunlight or incubated with secondary antibodies 2'Ab-CW800 (middle row) or 2'Ab-DyLight800 (bottom row) and kept in the dark (no sunlight). Scale bar = 30 μm.

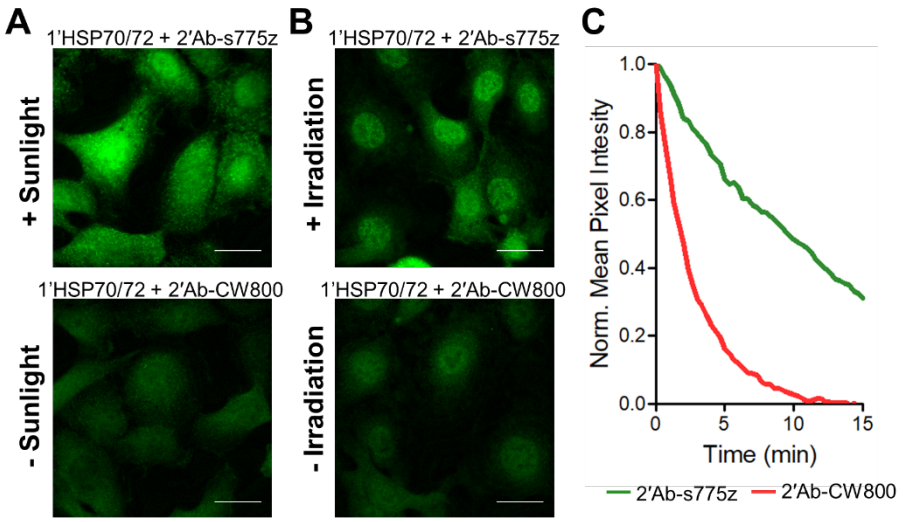


Figure S11. Photostability comparison of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes for targeting to primary anti-HSP70/72 in fixed cells. HT-1080 cells were fixed, incubated with anti-HSP70/72 overnight at 4 °C, and incubated the following day with fluorescent secondary antibody (2'Ab-s775z, 2'Ab-CW800) for 2 hr at room temperature. Representative epifluorescence cell micrographs of cells either (A) placed in the sunlight for 16 hr or (B) continuously irradiated for 15 min with a microscope equipped with an ICG filter (ex: 769/41, em: 832/37). (C) Plot of the mean pixel intensity of time-lapse cell micrographs captured at 10-20 sec time intervals during the 15-min irradiation with the microscope. Values were normalized to mean pixel intensity at time = 0 min. Scale bar = 30 μm.

Table S2. Half-life and rate constant of the first-order fluorescence decay due to photobleaching. Cells were incubated with primary antibody and secondary goat anti-rabbit IgG antibodies labeled with NIR dyes and continuously irradiated within a microscope equipped with an ICG filter (ex: 769/41, em: 832/37).

Cell Type :Protein of Interest	Fluorescent Secondary Antibody	Half-life $t_{1/2}$ (min)	Rate Constant k (min^{-1})	Decay Rate Compared to 2'Ab-s775z
HT-1080: α/β-Tubulin				
	2'Ab-s775z	8.81	0.079	
	2'Ab-CW800	2.71	0.256	3.3
	2'Ab-DyLight800	2.53	0.274	3.5
U87-MG: α/β-Tubulin				
	2'Ab-s775z	12.0	0.058	
	2'Ab-CW800	2.10	0.331	5.7
	2'Ab-DyLight800	3.21	0.216	3.7
A549: α/β-Tubulin				
	2'Ab-s775z	8.76	0.080	
	2'Ab-CW800	2.06	0.337	4.3
	2'Ab-DyLight800	3.26	0.213	2.7
HT-1080: HSP70/72				
	2'Ab-s775z	9.26	0.075	
	2'Ab-CW800	1.91	0.364	4.8

6. Immunohistochemistry

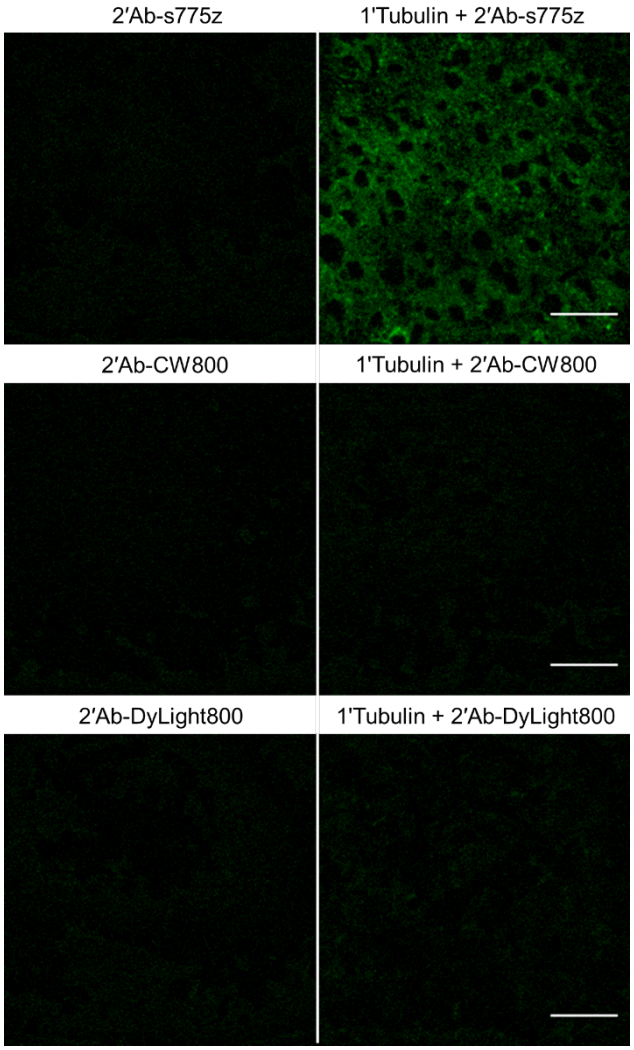
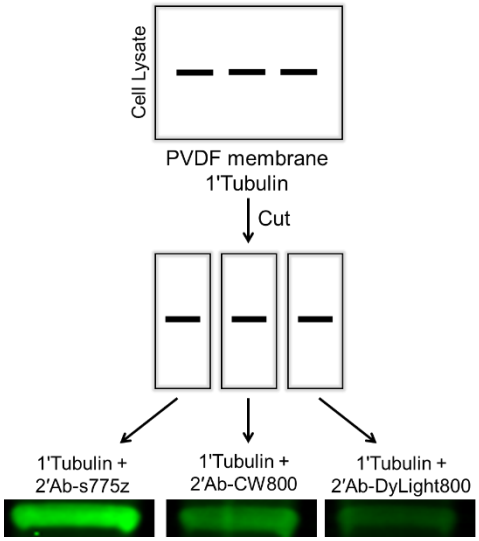


Figure S12. Control experiment for immunohistochemistry. Each mouse brain tissue slice was incubated with fluorescent secondary antibody (2'Ab-s775z, 2'Ab-CW800, 2'Ab-DyLight800) for 2 hr at room temperature and imaged using the same microscopic NIR fluorescence acquisition parameters employed in Figure 4. There is minimal non-specific staining of the tissue when the primary anti-Tubulin is absent. Scale bar = 1 mm.

7. Western Blotting



Scheme S2. Workflow for western blots experiments. HT-1080 cell lysate was aliquoted equally into three lanes on a polyacrylamide SDS gel, and after protein separation, the protein bands were transferred to a polyvinylidene fluoride membrane. The membrane was incubated with primary anti-Tubulin overnight at 4 °C and cut into three pieces, each containing an identical cell lysate lane. Each membrane piece was then incubated with one of the fluorescent secondary antibodies (2'Ab-s775z, 2'Ab-CW800 or 2'Ab- DyLight800) for 1 hr at room temperature, and a NIR fluorescence image of each α/β-Tubulin band was acquired using a LI-COR Odyssey Infrared Imaging System equipped with an 800 nm detection channel.

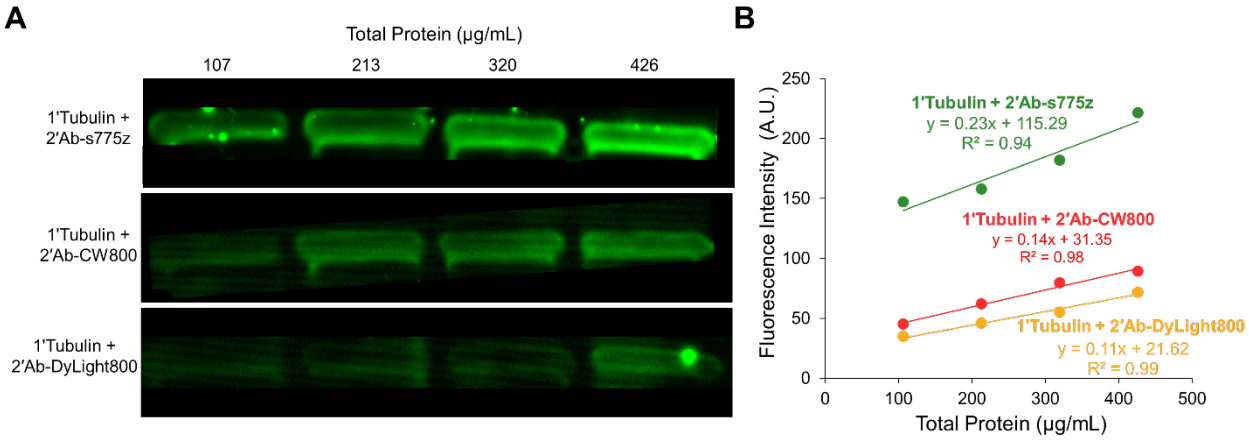
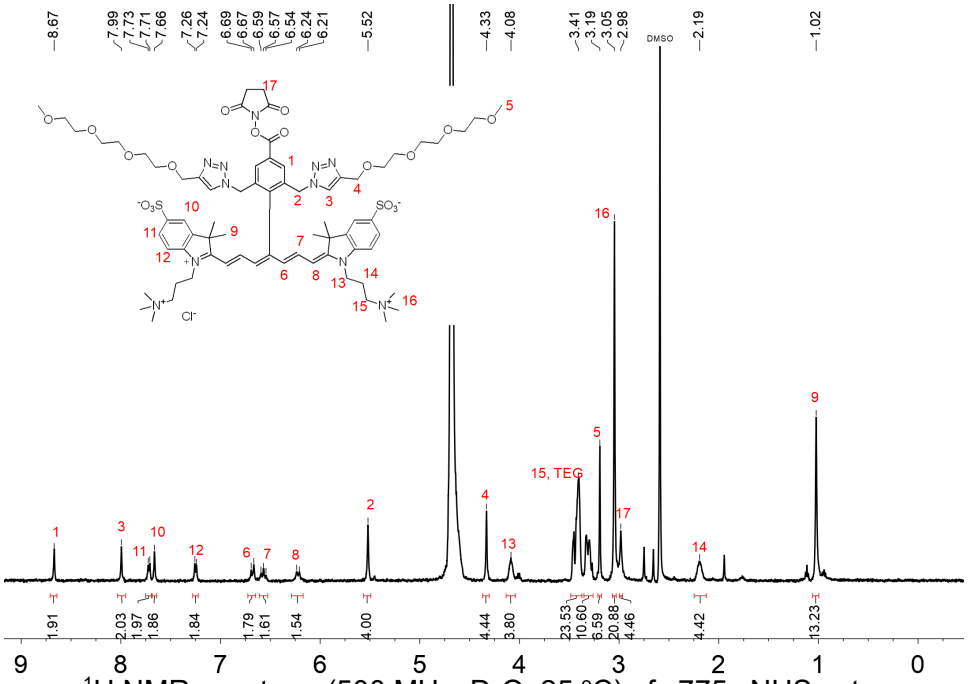
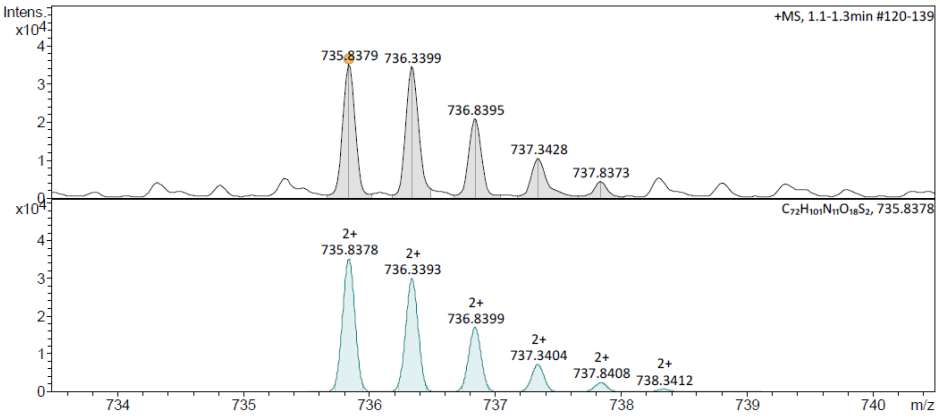


Figure S13. Comparison of secondary goat anti-rabbit IgG antibodies labeled with NIR dyes for targeting primary anti-Tubulin in cell lysate. Varying concentrations of HT-1080 cell lysate were loaded and run on SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated with anti-Tubulin overnight at 4 °C and then incubated with one of the fluorescent secondary antibodies (2'Ab-s775z, 2'Ab-CW800, or 2'Ab-DyLight800) for 1 hr at room temperature. (A) Fluorescent image of α/β-Tubulin band at various concentrations of total intracellular protein for each fluorescent secondary antibody. (B) Plot of fluorescence intensity of α/β-Tubulin band vs total intracellular protein with calculated linear regression line and r-squared value for each fluorescent secondary antibody.

8. Characterization of s775z NHS ester



^{1}H NMR spectrum (500 MHz, D_2O, 25 °C) of s775z NHS ester



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
735.8379	1	C72H101N11O18S2	735.8378	-0.1	83.0	1	100.00	28.0	even	ok

HRMS (ESI-TOF) spectrum of s775z NHS ester